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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:	
Stewart COLE, Roland BUCHREISER-BROSCH, Stephen GORDON, and Alain BILLAULT	Group Art Unit: 1654
Application No.: 10/802,796	Examiner: Julie HA
Filed: March 18, 2004	
For: A METHOD FOR ISOLATING A POLYNUCLEOTIDE OF INTEREST FROM THE GENOME OF MYCOBACTERIUM USING A BAC-BASED DNA LIBRARY, APPLICATION TO THE DETECTION OF MYCOBACTERIA	Confirmation No.: 6307

Attention: Mail Stop Appeal Brief-Patents
Commissioner for Patents
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07/14/2008 JADD01 00000044 10802796
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Sir:

APPEAL BRIEF UNDER BOARD RULE § 41.37

In response to the Notice of Panel Decision mailed May 15, 2008, requiring an Appeal Brief to maintain the application's pendency, and further to Board Rule 41.37, Appellant presents this brief. Appellant also encloses a check for the fee of $510.00, as required under 37 C.F.R. § 1.17(c). This brief is being filed concurrently with a Petition for Extension of Time of one month and the fee of $120.00.

This Appeal responds to the October 17, 2007, final rejection of claims 51-54 and 57, all of which are set forth in the attached Appendix A. If any additional fees are required or if the enclosed payment is insufficient, Appellant requests that the required fees be charged to Deposit Account No. 06-0916.

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I. Real Party in Interest

The assignment recorded on November 30, 2000, at reel 011332, frame 0132 designates Institut Pasteur as the Assignee of record of parent U.S. Application No. 09/673,476, which matured into U.S. Patent No. 7,112,663. Institut Pasteur is, therefore, the real party in interest in this appeal.

II. Related Appeals and Interferences

There are currently no other appeals or interferences, of which Appellant, Appellant's legal representative, or Assignee are aware, that will directly affect, be directly affected by, or have a bearing on, the Board's decision in the pending appeal.

III. Claim Status

Claims 51-54 and 57 are pending and stand presently rejected. Claims 1-50 and 55-56 have been cancelled. [Appendix A]. Appellant now appeals the rejection of claims 51-54 and 57.

IV. Status of Amendments

Appellant filed an Amendment After Final Action, under 37 C.F.R. § 1.116, on February 19, 2008. The Examiner responded with an Advisory Action on March 17, 2008, entering the Amendment After Final Action. Appellant then filed a Pre-appeal Brief Request for Review, accompanied by a Notice of Appeal, on April 15, 2008.

V. Summary of Claimed Subject Matter

Appellant claims polypeptides encoded by polynucleotides present in a fragment of the *Mycobacterium tuberculosis* genome but not in the *Mycobacterium bovis* genome. The presence or absence of these polypeptides in a biological sample determines whether *M. tuberculosis* is present in the sample, regardless of whether or not *M. bovis*

is present. Testing for the presence or absence of these peptides can distinguish individuals pathologically infected with *M. tuberculosis* from those intentionally vaccinated with *M. bovis*.

Appellant cloned bacterial DNA in a Bacterial Artificial Chromosome ("BAC"), then identified the polynucleotides that encode the claimed polypeptides in an *M. tuberculosis* genomic DNA library. [WO 99/54487 ("Specification") at p. 2, l. 16-17; p. 3, l. 29-35; p. 6, l. 3-6; p. 32, l. 19-35]. By cloning the H37Rv strain of *M. tuberculosis* into a pBeloBAC11 vector, Appellant produced a library that covered 97% of the H37Rv tuberculosis genome with BAC clones. [Specification at p. 32, l. 9-35; p. 34, l. 2-3].

Appellant then performed comparative genomics between different strains of mycobacteria with clones from the library, pinpointing polymorphic regions in the mycobacterial genomes. Restriction fragments that failed to hybridize with a different strain were identified as absent from the genome of that strain. [Specification at p. 36, l. 30-31]. Using this approach, Appellant identified polynucleotides differentially expressed in mycobacterial strains.

In particular, several restriction fragments from the *M. tuberculosis* BAC clones failed to hybridize with genomic DNA from *M. bovis*. [Specification at p. 37, l. 5-6]. "*M. tuberculosis, M. bovis* and *M. bovis* BCG [an attenuated culture of *M. bovis*], specifically BCG Pasteur strain, exhibit a high level of global genomic conservation, but certain polymorphic regions were also detected." [Specification at p. 6, l. 28-30]. "[R]estriction fragments that fail to hybridize with the *M. bovis* BCG Pasteur DNA are absent from its

genome, hence identifying polymorphic regions between *M. bovis* BCG Pasteur and *M. tuberculosis* H37Rv." [Specification at p. 36, l. 30-34; Figure 3].

Aligning the *M. tuberculosis* H37Rv DNA sequences with the *M. bovis* and *M. bovis* BCG Pasteur strains identified 12,732 base pairs (*i.e.*, approximately 12.7 kb) present in the former and absent in the latter two strains. [Specification at p. 37, l. 11-13]. Subsequent PCR studies showed that this segment was also absent from Danish, Russian, and Glaxo substrains of *M. bovis* BCG, demonstrating that this polymorphism can be generally used to distinguish *M. tuberculosis* from *M. bovis* bacterial strains. [Specification at p. 37, l. 17-20].

This 12.7 kb polynucleotide (SEQ ID NO: 1) corresponds to nucleotides 1696015 to 1708746 of the *M. tuberculosis* genome and comprises eleven predicted open reading frames ("ORFs"). [Specification at p. 5, l. 8-14]. SEQ ID NO: 1, or its fragments, can be used to screen biological samples to determine whether they comprise *M. tuberculosis* or *M. bovis* strains. This polynucleotide can be detected by bringing the sample into contact with BAC vectors or purified polynucleotides of the invention, and detecting the hybrid complexes formed. [Specification at p. 25, l. 33 to p. 26, l. 4]. The presence or absence of SEQ ID NO: 1 or its fragments in a biological sample indicates whether the sample came from an individual infected with *M. tuberculosis* or vaccinated with *M. bovis*.

SEQ ID NO: 1 comprises eleven open reading frames designated MTCY277.28 to MTCY277.38, and deposited as EMBL accession number Z79701. This

polynucleotide is illustrated as a diagram in Figure A, shown below, with the ORFs labeled 1-11 and the numeral location in the genome indicated for each.

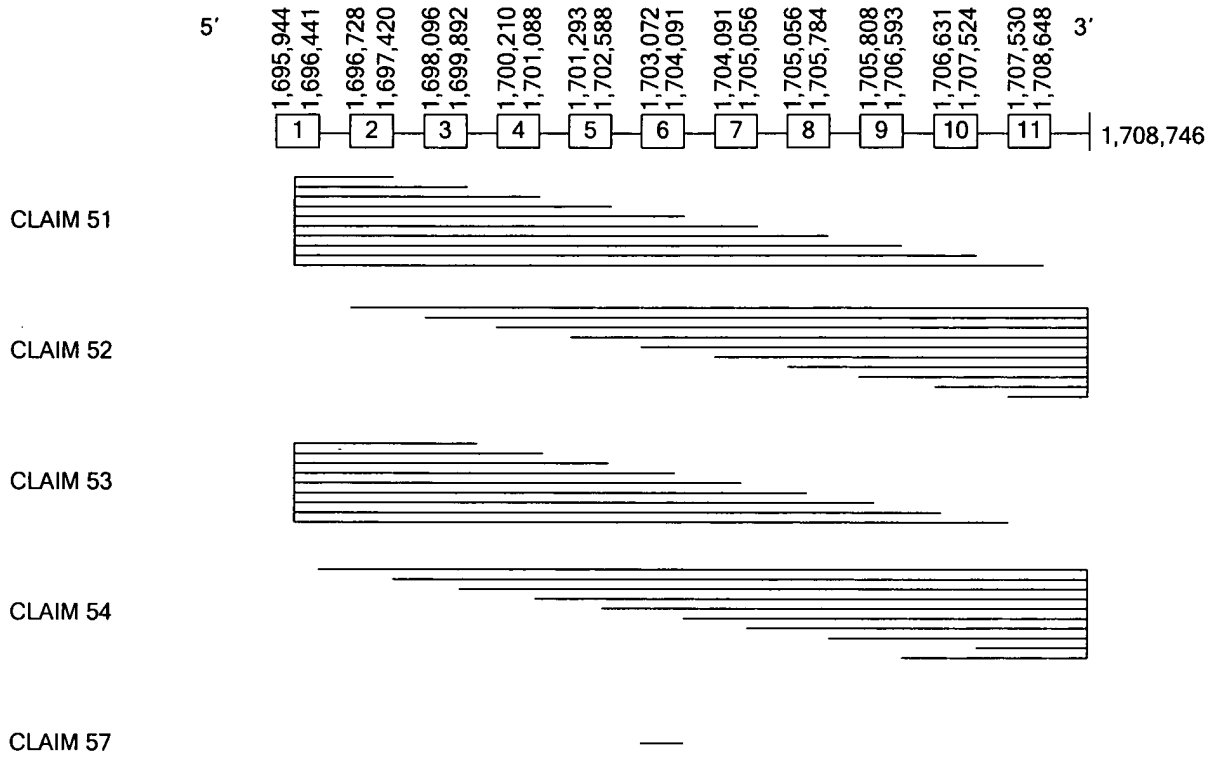


Figure A

Protein database searches indicate that the claimed polypeptides encoded by this region of the genome may be involved in the biosynthesis of polysaccharides, major components of the mycobacterial cell wall. [Specification at p. 9, l. 27-28]. Differences in the proteins that regulate polysaccharide biosynthesis reflect differences in cell wall composition. Detecting these differences "is of diagnostic interest[,]" as they define structural differences between *M. tuberculosis* and *M. bovis* strains. [Specification at p. 9, l. 25 to p. 10, l. 2]. Polynucleotides "encoding all or part of [the claimed] polypeptide[s

are] involved in an important metabolic[al] and/or physiological pathway of the mycobacteria." [Specification at p. 10, l. 5-6]. Using conventional methods, the claimed polypeptides can detect *M. tuberculosis*, but not *M. bovis*, and are thus useful in diagnostic tests performed to distinguish an immune response induced by vaccination with *M. bovis* from one induced by infection with *M. tuberculosis*.

VI. The Appealed Claims

A. Identification of Claims with Reference to the Specification as Filed on March 18, 2004

Claim No.	Claim	Corresponding Disclosure
51	A purified polypeptide, encoded by a polynucleotide comprising an ORF contained within SEQ ID NO: 1, wherein the polynucleotide is selected from the following nucleotides of the *M. tuberculosis* chromosome:	page 12, line 8 to page 13, line 5
	a. nucleotide 1,696,019 through nucleotide 1,697,420	page 12, line 12; page 12, line 15
	b. nucleotide 1,696,019 through nucleotide 1,699,892	page 12, line 12; page 12, line 17
	c. nucleotide 1,696,019 through nucleotide 1,701,088	page 12, line 12; page 12, line 20
	d. nucleotide 1,696,019 through nucleotide 1,702,588	page 12, line 12; page 12, line 22
	e. nucleotide 1,696,019 through nucleotide 1,704,091	page 12, line 12; page 12, line 25
	f. nucleotide 1,696,019 through nucleotide 1,705,056	page 12, line 12; page 12, line 28
	g. nucleotide 1,696,019 through nucleotide 1,705,784	page 12, line 12; page 12, line 32

Claim No.	Claim	Corresponding Disclosure
	h. nucleotide 1,696,019 through nucleotide 1,706,593	page 12, line 12; page 12, line 34
	i. nucleotide 1,696,019 through nucleotide 1,707,524	page 12, line 12; page 13, line 2
	j. nucleotide 1,696,019 through nucleotide 1,708,648.	page 12, line 12; page 13, line 4
52	A purified polypeptide, encoded by a polynucleotide comprising an ORF contained within SEQ ID NO: 1, wherein the polynucleotide is selected from the following nucleotides of the *M. tuberculosis* chromosome:	page 12, line 8 to page 13, line 5
	a. nucleotide 1,696,728 through nucleotide 1,708,746	page 12, line 14; page 10, lines 34-35
	b. nucleotide 1,698,096 through nucleotide 1,708,746	page 12, line 16; page 10, lines 34-35
	c. nucleotide 1,700,210 through nucleotide 1,708,746	page 12, line 19; page 10, lines 34-35
	d. nucleotide 1,701,293 through nucleotide 1,708,746	page 12, line 21; page 10, lines 34-35
	e. nucleotide 1,703,072 through nucleotide 1,708,746	page 12, line 24; page 10, lines 34-35
	f. nucleotide 1,704,091 through nucleotide 1,708,746	page 12, line 27; page 10, lines 34-35
	g. nucleotide 1,705,056 through nucleotide 1,708,746	page 12, line 31; page 10, lines 34-35
	h. nucleotide 1,705,808 through nucleotide 1,708,746	page 12, line 33; page 10, lines 34-35
	i. nucleotide 1,706,631 through nucleotide 1,708,746	page 13, line 1; page 10, lines 34-35

Claim No.	Claim	Corresponding Disclosure
	j. nucleotide 1,707,530 through nucleotide 1,708,746.	page 13, line 3; page 10, lines 34-35
53	A purified polypeptide, encoded by a polynucleotide comprising an ORF contained within SEQ ID NO: 1, wherein the polynucleotide is selected from the following nucleotides of the *M. tuberculosis* chromosome:	page 12, line 8 to page 13, line 5
	a. nucleotide 1,696,019 through nucleotide 1,698,096	page 12, line 12; page 12, line 16
	b. nucleotide 1,696,019 through nucleotide 1,700,210	page 12, line 12; page 12, line 19
	c. nucleotide 1,696,019 through nucleotide 1,701,293	page 12, line 12; page 12, line 21
	d. nucleotide 1,696,019 through nucleotide 1,703,072	page 12, line 12; page 12, line 24
	e. nucleotide 1,696,019 through nucleotide 1,704,091	page 12, line 12; page 12, line 27
	f. nucleotide 1,696,019 through nucleotide 1,705,056	page 12, line 12; page 12, line 31
	g. nucleotide 1,696,019 through nucleotide 1,705,808	page 12, line 12; page 12, line 33
	h. nucleotide 1,696,019 through nucleotide 1,706,631	page 12, line 12; page 13, line 1
	i. nucleotide 1,696,019 through nucleotide 1,707,530.	page 12, line 12; page 13, line 3
54	A purified polypeptide, encoded by a polynucleotide comprising an ORF contained within SEQ ID NO: 1, wherein the polynucleotide is selected from the following nucleotides of the *M. tuberculosis* chromosome:	page 12, line 8 to page 13, line 5

Claim No.	Claim	Corresponding Disclosure
	a. nucleotide 1,696,441 through nucleotide 1,708,746	page 12, line 13; page 10, lines 34-35
	b. nucleotide 1,697,420 through nucleotide 1,708,746	page 12, line 15; page 10, lines 34-35
	c. nucleotide 1,699,892 through nucleotide 1,708,746	page 12, line 17; page 10, lines 34-35
	d. nucleotide 1,701,088 through nucleotide 1,708,746	page 12, line 20; page 10, lines 34-35
	e. nucleotide 1,702,588 through nucleotide 1,708,746	page 12, line 22; page 10, lines 34-35
	f. nucleotide 1,704,091 through nucleotide 1,708,746	page 12, line 25; page 10, lines 34-35
	g. nucleotide 1,705,056 through nucleotide 1,708,746	page 12, line 28; page 10, lines 34-35
	h. nucleotide 1,705,784 through nucleotide 1,708,746	page 12, line 32; page 10, lines 34-35
	i. nucleotide 1,707,524 through nucleotide 1,708,746	page 13, line 2; page 10, lines 34-35
	j. nucleotide 1,706,593 through nucleotide 1,708,746.	page 12, line 34; page 10, lines 34-35
57	A purified polypeptide encoded by a polynucleotide, comprising an ORF contained within SEQ ID NO: 1, wherein the polynucleotide consists of ORF6 between nucleotide 1,703,072 through nucleotide 1,704,091.	page 12, lines 24-26

B. Appellant's Interpretation of Claim 51: A Purified Polypeptide Encoded by a Polynucleotide Comprising an ORF Contained Within SEQ ID NO: 1, Selected From a Group of Polypeptides Encoded by Nested Polynucleotides Beginning at a Nucleotide Near the 5' End of SEQ ID NO: 1 and Ending at the 3' End of ORFs 2-11

Claim 51 recites a purified polypeptide, encoded by a polynucleotide comprising an ORF contained within SEQ ID NO: 1, wherein the polynucleotide is selected from the 12.7 kb nucleotide sequence (SEQ ID NO: 1) present in the *M. tuberculosis* chromosome but absent from *M. bovis*. [Specification at p. 12, l. 8 to p. 13, l. 5]. This claim recites a group of nested polypeptides encoded by polynucleotides, beginning at nucleotide 1,696,019, in ORF1. The shortest member of the group, recited by claim 51a, ends at the final nucleotide of ORF2 and the members grow successively longer to sequentially include ORFs, 3-11 as illustrated in Figure A, shown above.

C. Appellant's Interpretation of Claim 52: A Purified Polypeptide Encoded by a Polynucleotide Comprising an ORF Contained Within SEQ ID NO: 1, Selected From a Group of Polypeptides Encoded by Nested Polynucleotides Beginning at the 5' End of ORFs 2-11 and Ending at the 3' Nucleotide of SEQ ID NO: 1

Claim 52 also recites a purified polypeptide, encoded by a polynucleotide comprising an ORF contained within SEQ ID NO: 1, wherein the polynucleotide is selected from the 12.7 kb nucleotide sequence (SEQ ID NO: 1) present in the *M. tuberculosis* chromosome but absent from *M. bovis*. [Specification at p. 12, l. 8 to p. 13, l. 5]. This claim recites a group of nested polypeptides encoded by polynucleotides, beginning at nucleotide 1,696,728, corresponding to the 5' nucleotide of ORF2, and ending at nucleotide 1,708,746, the 3' nucleotide of SEQ ID NO: 1. The longest member of this group, recited by claim 52a, begins at the 5' nucleotide of ORF2 and the

members grow successively shorter to sequentially exclude ORFs 2-10, as illustrated in Figure A.

D. Appellant's Interpretation of Claim 53: A Purified Polypeptide Encoded by a Polynucleotide Comprising an ORF Contained Within SEQ ID NO: 1, Selected From a Group of Polypeptides Encoded by Nested Polynucleotides Beginning at a Nucleotide Near the 5' End of SEQ ID NO: 1 and Ending at the 5' Nucleotide of ORFs 3-11

Claim 53 also recites a purified polypeptide, encoded by a polynucleotide comprising an ORF contained within SEQ ID NO: 1, wherein the polynucleotide is selected from the 12.7 kb nucleotide sequence (SEQ ID NO: 1) present in the *M. tuberculosis* chromosome but absent from *M. bovis*. [Specification at p. 12, l. 8 to p. 13, l. 5]. The claim recites a group of nested polypeptides encoded by polynucleotides, beginning at nucleotide 1,696,019, in ORF1. The shortest member of the group, recited by claim 53a, ends at the 5' nucleotide of ORF3 and the members grow successively longer to sequentially include the intron following ORFs 3-10, as illustrated in Figure A.

E. Appellant's Interpretation of Claim 54: A Purified Polypeptide Encoded by a Polynucleotide Comprising an ORF Contained Within SEQ ID NO: 1, Selected From a Group of Polypeptides Encoded by Nested Polynucleotides Beginning at the 3' End of ORFs 1-11 and Ending at the 3' Nucleotide of SEQ ID NO: 1

Claim 54 also recites a purified polypeptide, encoded by a polynucleotide comprising an ORF contained within SEQ ID NO: 1, wherein the polynucleotide is selected from the 12.7 kb nucleotide sequence (SEQ ID NO: 1) present in the *M. tuberculosis* chromosome but absent from *M. bovis*. [Specification at p. 12, l. 8 to p. 13, l. 5]. This claim recites a group of nested polypeptides encoded by polynucleotides, beginning at nucleotide 1,696,728, corresponding to the 5' nucleotide of ORF2, and ending at the 3' nucleotide of SEQ ID NO: 1. The longest member of this group, recited

by claim 54a, begins at the 3' nucleotide of ORF1 and the members grow successively shorter to sequentially exclude ORFs 2-10, as illustrated in Figure A.

F. Appellant's Interpretation of Claim 57: A Purified Polypeptide Encoded by a Polynucleotide Comprising ORF6

Claim 57 recites a purified polypeptide, encoded by a polynucleotide comprising an ORF contained within SEQ ID NO: 1, wherein the polynucleotide consists of ORF6, located between nucleotide 1,703,072 and nucleotide 1,704,091, as shown in Figure A. [Specification at p. 12, l. 24-25]. ORF6, the gene within the 12.7 kb fragment with the greatest homology to a known gene, encodes a protein with characteristics of a GDP D-mannose dehydratase. [Specification at p. 12, l. 25-26; p. 37, l. 24-28].

VII. Grounds of Rejection to Be Reviewed on Appeal

A. Claims 51-54 and 57 Allegedly Lack Utility, Under 35 U.S.C. § 101

The Examiner rejected claims 51-54 and 57 for lack of utility, under 35 U.S.C. §101, entirely on the basis of the Examiner's allegation that the specification does not disclose polypeptides with GDP-D-mannose dehydratase activity. The Examiner supported the rejection by asserting that the claimed polypeptides share only 51% homology with known GDP-D-mannose dehydratases. [Final Office Action of October 17, 2007, at p. 2]. The Examiner did not address Appellant's assertion that the claimed polypeptides are useful for distinguishing mycobacterial strains.

B. Claims 51-54 and 57 Allegedly Are Not Enabled, Under 35 U.S.C. § 112, ¶ 1

Claims 51-54 and 57 also stand rejected under 35 U.S.C. § 112, first paragraph, for allegedly failing to comply with the enablement requirement because the invention is not supported by a substantial utility. Thus, according to the Examiner, one skilled in

the art would not know how to use the claimed invention, without undue experimentation. [Final Office Action of October 17, 2007, at p. 4-5].

VIII. Argument

A. Appellant Asserted that the Claimed Polypeptides Possess the Specific, Substantial, and Credible Utility of Distinguishing *M. Tuberculosis* From *M. Bovis*

1. The Claimed Polypeptides Distinguish *M. Tuberculosis* From *M. Bovis*

Under the Office's guidelines for determining compliance with the utility requirement, under 35 U.S.C. § 101, a claimed invention must be supported by a "specific and substantial utility that is credible[.]" [Exhibit 1: Utility Examination Guidelines, 66 Fed. Reg. 1092, 1098 (2001)]. A utility specific for the claimed polypeptides is one attributed to those particular polypeptides, in contrast to a general utility, which could be ascribed to "polypeptides" as a class. [Exhibit 2: Revised Interim Utility Guidelines Training Materials, Synopsis, p. 3, 5 (1999)]. A substantial utility for these polypeptides entails a real world use. [*Id.*, at 6]. Finally, the asserted utility is considered credible unless the logic underlying the assertion is seriously flawed or the facts upon which the assertion is based are inconsistent with the underlying logic. [*Id.*, at 5].

Infection with the bacterium *M. tuberculosis* causes tuberculosis, a contagious and potentially fatal disease of humans. *M. bovis*, a strain of the bacteria that infects cattle, is commonly used to vaccinate humans. These two strains are quite similar, with the exception of the genomic fragment identified by Appellant. Distinguishing them in a

biological sample identifies individuals infected with *M. tuberculosis* as distinct from those vaccinated with *M. bovis*.

Appellant asserted that the claimed polypeptides are useful for distinguishing *M. tuberculosis* from *M. bovis*. They "perform[ed] comparative genomics between different strains or species of mycobacterial cells," and "identifi[ed] polymorphic regions between *M. bovis* BCG Pasteur and *M. tuberculosis* H37Rv." [Specification at p. 9, l. 4-21]. One of these polymorphic regions, a 12.7 kb region of SEQ ID NO:1, contains eleven open reading frames, *i.e.*, extended sequences of codons translated during mRNA synthesis, and free of stop codons such that they can transcribe polypeptides. [Specification at p. 9, l. 25-27].

As stated above, "Assuming that some of the gene products from this region represent proteins with antigenic properties, it could be possible to develop a test that can reliably distinguish between the immune response induced by vaccination with *M. bovis* BCG vaccine strains and infection with *M. tuberculosis*." [Specification at p. 11, l. 6-10]. According to the Office, this assumption is well-grounded. "Early studies empirically established that proteins were good antigens when injected into a species other than the one from which they originated." [Written Description Training Materials, Revision 1, March 25, 2008, Example 13, p. 45, citing Kabat, EA, *Structural Concepts in Immunology and Immunochemistry, 2nd Ed,* (1976)]. The claimed proteins act as antigens, which can be detected by conventional immunoassays. Their presence indicates the presence of *M. tuberculosis* regardless of whether or not *M. bovis* is present in the sample.

The clamed polypeptides are specific to the polymorphic region of the mycobacterial genome, which distinguishes one strain from another. It is precisely these polypeptides that provide a target for distinguishing *M. tuberculosis* in a sample also comprising *M. bovis*. While the two strains "exhibit a high level of global genomic conservation," the novel 12.7 kb segment present in *M. tuberculosis* but absent from *M. bovis* distinguishes these mycobacterial species. [Specification at p. 6, l. 28]. Thus, these particular polypeptides provide a means to distinguish the two strains.

The claimed polypeptides have a substantial, real world, utility simply as a consequence of their differential expression by mycobacterial strains. More than two billion people worldwide have been immunized with the attenuated *M. bovis* BCG and test positive in assays for the presence of mycobacteria. [Exhibit 4: Philipp, WJ *et al.*, Physical mapping of *Mycobacterium bovis* BCG Pasteur reveals differences from the genome map of *Mycobacterium tuberculosis* H37Rv and from *M. bovis*, *Microbiology* 142:3135-45 (1996) at 3135; considered by the Examiner May 5, 2003]. These vaccinated subjects do not, however, test positive in assays for the claimed polypeptides unless they are also infected with *M. tuberculosis*. The claimed polypeptides can be detected in immunoassays designed to identify a subject infected with *M. tuberculosis*, who may also have been vaccinated with *M. bovis*.

This distinction can inform the choice of pharmacologic treatment protocols, for example, by more precisely identifying patients who would benefit from therapy for *M. tuberculosis*, and sparing unnecessary treatment of those uninfected. It also provides epidemiologists with a means for identifying the source of an infection. The

polypeptides of the invention can be used in the same comparative assays to distinguish patients infected from zoonotic sources of *M. bovis*, *e.g.,* unpasteurized dairy products and cattle-borne infection, from those who contracted their disease from a human source.

The facts asserted by Appellant, *e.g.*, that the claimed polypeptides are encoded by a region of the genome present in one strain but not the other, logically support the asserted utility of using them to distinguish *M. tuberculosis* from *M. bovis*. This utility is, therefore, credible, as one skilled in the art would understand that a comparative assay based on the ability to detect the claimed polypeptides can distinguish the bacterial strains.

Finally, it is critical to understand that the asserted utility is independent of any homology to GDP-D-mannose dehydratases. This utility derives from the selective presence of one or more claimed polypeptides in one strain of *Mycobacterium* compared to another strain, and the ability of a conventional assay to distinguish the two strains by the mere presence (or absence) of the polypeptide. [Reply to Final Office Action of February 19, 2008, at p. 2].

2. The Examiner Failed to Consider Appellant's Response to the Rejection

Claims 51-54 and 57 stand rejected, under 35 U.S.C. §101, for lack of utility, entirely on the basis of the Examiner's allegation that the specification does not disclose polypeptides with GDP-D-mannose dehydratase activity, because the claimed polypeptides share only 51% homology with known GDP-D-mannose dehydratases. [Final Office Action of October 17, 2007, at pp. 3-4]. Appellant has, however, traversed

the rejection on different grounds, set forth above in Section VIIIA1, which have not been considered on their merits by the Examiner. The Examiner acknowledged the existence of Appellant's argument but made no substantive response. "Applicant argues since the polypeptide is expressed by *M. tuberculosis* but not *M. bovis* BCG, one of skill in the art would have understood that the polypeptide of the invention would have utility to distinguish *M. bovis* BCG from *M. tuberculosis*." [*Id.*, at p. 3]. Nowhere in the record, however, does the Examiner counter this argument.

Appellant first traversed the rejection in the Reply to the First Office Action, presenting a reasoned argument. "[R]egardless of the function of the encoded polypeptide . . . the claimed polynucleotide sequence of the invention had utility simply as a consequence of its expression by *M. tuberculosis*, but not *M. bovis* BCG. [Reply to Office Action of March 12, 2007, at p. 4]. The Examiner upheld the rejection in the Final Action, without acknowledging that the utility of distinguishing the two bacterial strains does not relate to the enzymatic dehydratase activity of the claimed polypeptide, nor to its homology to known dehydratases.

Appellant again traversed, providing additional support for its position by stating that the claimed polypeptides can be used in diagnostic immunoassays to distinguish a subject infected with *M. tuberculosis* from a subject vaccinated against *M. tuberculosis* with an *M. bovis* vaccine. [Reply to Final Office Action of February 19, 2008, at p. 2]. The presence or absence of a particular genomic region can identify the presence or absence of *M. tuberculosis or M. bovis*. This ability to distinguish between these two *Mycobacterium* strains is a substantial and well-established utility. [*Id.*, at pp. 2-3].

The Advisory Action of March 17, 2008, maintained the rejections of claims 51-54 and 57, "[f]or the reasons set forth in the previous [O]ffice [A]ction." [Advisory Action of March 17, 2008, at p. 2]. The record reflects that the Examiner's rationale for these rejections is a lack of homology with a known protein. It also reflects that Appellant's previous arguments stating that the claimed polypeptides are useful because they can distinguish between mycobacterial strains have not been addressed.

The record, therefore, reflects that the Examiner acted improperly by failing to consider Appellant's reasoning in support of an asserted specific and substantial credible utility, as required. [M.P.E.P. § 2107.02]. "It is essential for Office personnel to recognize, fully consider and respond to each substantive element of any response to a rejection based on lack of utility." [*Id.*, emphasis added]. Appellant requests that the Board now consider the totality of the record and find that the claimed invention meets all requirements for utility set forth in 35 U.S.C. § 101.

B. The Enablement Rejection Is Improper Because the Utility Rejection Is Improper

The enablement rejection is contingent on an improper utility rejection, according to the provision of the M.P.E.P. regarding the relationship between 35 U.S.C. § 101 and §112, which reads as follows:

> Office personnel should not impose a 35 U.S.C. § 112, first paragraph rejection grounded on a "lack of utility" basis unless a 35 U.S.C. § 101 rejection is proper.

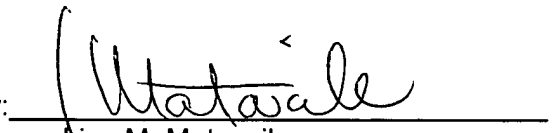
[M.P.E.P. § 2107.01]. Appellant therefore respectfully asserts that the rejection of claims 51-54 and 57, under 35 U.S.C. § 112, first paragraph, must be withdrawn concurrently with the withdrawal of the rejection under 35 U.S.C. § 101.

IX. Conclusion

For the reasons given above, Appellant respectfully requests that the Board reverse all grounds for rejection of claims 51-54 and 57.

FINNEGAN, HENDERSON, FARABOW,
GARRETT & DUNNER, L.L.P.

Dated: July 11, 2008

By: 

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APPENDIX A

CLAIMS APPENDIX TO APPEAL BRIEF UNDER RULE 41.37(C)(1)(VIII)

The following list of claims reflects the most recent amendment of the claims, filed December 13, 2006, and entered March 12, 2007.

1.-50. (Canceled)

51. (Previously Presented) A purified polypeptide, encoded by a polynucleotide comprising an Open Reading Frame contained within SEQ ID NO: 1, wherein the polynucleotide is selected from:

(a) nucleotide 1,696,019 through nucleotide 1,697,420 of the Mycobacterium tuberculosis chromosome;

(b) nucleotide 1,696,019 through nucleotide 1,699,892 of the Mycobacterium tuberculosis chromosome;

(c) nucleotide 1,696,019 through nucleotide 1,701,088 of the Mycobacterium tuberculosis chromosome;

(d) nucleotide 1,696,019 through nucleotide 1,702,588 of the Mycobacterium tuberculosis chromosome;

(e) nucleotide 1,696,019 through nucleotide 1,704,091 of the Mycobacterium tuberculosis chromosome;

(f) nucleotide 1,696,019 through nucleotide 1,705,056 of the Mycobacterium tuberculosis chromosome;

(g) nucleotide 1,696,019 through nucleotide 1,705,784 of the Mycobacterium tuberculosis chromosome;

(h) nucleotide 1,696,019 through nucleotide 1,706,593 of the Mycobacterium tuberculosis chromosome;

(i) nucleotide 1,696,019 through nucleotide 1,707,524 of the Mycobacterium tuberculosis chromosome; or

(j) nucleotide 1,696,019 through nucleotide 1,708,648 of the Mycobacterium tuberculosis chromosome.

52. (Previously Presented) A purified polypeptide, encoded by a polynucleotide, comprising an Open Reading Frame contained within SEQ ID NO: 1, wherein the polynucleotide is selected from:

(a) nucleotide 1,696,728 through nucleotide 1,708,746 of the *Mycobacterium tuberculosis* chromosome;

(b) nucleotide 1,698,096 through nucleotide 1,708,746 of the *Mycobacterium tuberculosis* chromosome;

(c) nucleotide 1,700,210 through nucleotide 1,708,746 of the *Mycobacterium* tuberculosis chromosome;

(d) nucleotide 1,701,293 through nucleotide 1,708,746 of the Mycobacterium tuberculosis chromosome;

(e) nucleotide 1,703,072 through nucleotide 1,708,746 of the Mycobacterium tuberculosis chromosome;

(f) nucleotide 1,704,091 through nucleotide 1,708,746 of the Mycobacterium tuberculosis chromosome;

(g) nucleotide 1,705,056 through nucleotide 1,708,746 of the Mycobacterium tuberculosis chromosome;

(h) nucleotide 1,705,808 through nucleotide 1,708,746 of the Mycobacterium tuberculosis chromosome;

(i) nucleotide 1,706,631 through nucleotide 1,708,746 of the Mycobacterium tuberculosis chromosome; or

(j) nucleotide 1,707,530 through nucleotide 1,708,746 of the Mycobacterium tuberculosis chromosome.

53. (Previously Presented) A purified polypeptide, encoded by a polynucleotide, comprising an Open Reading Frame contained within SEQ ID NO: 1, wherein the polynucleotide is selected from:

(a) nucleotide 1,696,019 through nucleotide 1,698,096 of the *Mycobacterium tuberculosis* chromosome;

(b) nucleotide 1,696,019 through nucleotide 1,700,210 of the *Mycobacterium tuberculosis* chromosome;

(c) nucleotide 1,696,019 through nucleotide 1,701,293 of the Mycobacterium tuberculosis chromosome;

(d) nucleotide 1,696,019 through nucleotide 1,703,072 of the Mycobacterium tuberculosis chromosome;

(e) nucleotide 1,696,019 through nucleotide 1,704,091 of the Mycobacterium tuberculosis chromosome;

(f) nucleotide 1,696,019 through nucleotide 1,705,056 of the Mycobacterium tuberculosis chromosome;

(g) nucleotide 1,696,019 through nucleotide 1,705,808 of the Mycobacterium tuberculosis chromosome;

(h) nucleotide 1,696,019 through nucleotide 1,706,631 of the Mycobacterium tuberculosis chromosome; or

(i) nucleotide 1,696,019 through nucleotide 1,707,530 of the Mycobacterium tuberculosis chromosome.

54. (Previously Presented) A purified polypeptide, encoded by a polynucleotide, comprising an Open Reading Frame contained within SEQ ID NO: 1, wherein the polynucleotide is selected from:

(a) nucleotide 1,696,441 through nucleotide 1,708,746 of the *Mycobacterium tuberculosis* chromosome;

(b) nucleotide 1,697,420 through nucleotide 1,708,746 of the *Mycobacterium* tuberculosis chromosome;

(c) nucleotide 1,699,892 through nucleotide 1,708,746 of the *Mycobacterium tuberculosis* chromosome;

(d) nucleotide 1,701,088 through nucleotide 1,708,746 of the Mycobacterium tuberculosis chromosome;

(e) nucleotide 1,702,588 through nucleotide 1,708,746 of the Mycobacterium tuberculosis chromosome;

(f) nucleotide 1,704,091 through nucleotide 1,708,746 of the Mycobacterium tuberculosis chromosome;

(g) nucleotide 1,705,056 through nucleotide 1,708,746 of the Mycobacterium tuberculosis chromosome;

(h) nucleotide 1,705,784 through nucleotide 1,708,746 of the Mycobacterium tuberculosis chromosome;

(i) nucleotide 1,707,524 through nucleotide 1,708,746 of the Mycobacterium tuberculosis chromosome; or

(j) nucleotide 1,706,593 through nucleotide 1,708,746 of the Mycobacterium tuberculosis chromosome.

55-56. (Canceled)

57. (Previously Presented). A purified polypeptide encoded by a polynucleotide, comprising an Open Reading Frame contained within SEQ ID NO: 1, wherein the polynucleotide consists of ORF6 between nucleotide 1,703,072 through nucleotide 1,704,091.

APPENDIX B

EVIDENCE APPENDIX TO APPEAL BRIEF UNDER RULE 41.37(C)(1)(IX)

Exhibit 1: Utility Examination Guidelines, 66 Fed. Reg. 1092, 1098 (2001)

Exhibit 2: Revised Interim Utility Guidelines Training Materials, Synopsis, p. 3-8 (1999).

Exhibit 3: Written Description Training Materials, Revision 1, March 25, 2008, Example 13, p. 45, citing Kabat, EA, *Structural Concepts in Immunology and Immunochemistry, 2nd Ed,* (1976).

Exhibit 4: Philipp, WJ *et al.*, Physical mapping of *Mycobacterium bovis* BCG Pasteur reveals differences from the genome map of *Mycobacterium tuberculosis* H37Rv and from *M. bovis*, *Microbiology* 142:3135-45 (1996), considered by the Examiner May 5, 2003.

Exhibit 1

requirement. The Guidelines do not alter the substantive requirements of 35 U.S.C. 101 and 112, nor are they designed to obviate the examiner's review of applications for compliance with all other statutory requirements for patentability. The Guidelines do not constitute substantive rulemaking and hence do not have the force and effect of law. Rejections will be based upon the substantive law, and it is these rejections which are appealable. Consequently, any perceived failure by Office personnel to follow these Guidelines is neither appealable nor petitionable.

B. Examination Guidelines for the Utility Requirement

Office personnel are to adhere to the following procedures when reviewing patent applications for compliance with the "useful invention" ("utility") requirement of 35 U.S.C. 101 and 112, first paragraph.

1. Read the claims and the supporting written description.

(a) Determine what the applicant has claimed, noting any specific embodiments of the invention.

(b) Ensure that the claims define statutory subject matter (*i.e.*, a process, machine, manufacture, composition of matter, or improvement thereof).

(c) If at any time during the examination, it becomes readily apparent that the claimed invention has a well-established utility, do not impose a rejection based on lack of utility. An invention has a well-established utility (1) if a person of ordinary skill in the art would immediately appreciate why the invention is useful based on the characteristics of the invention (*e.g.*, properties or applications of a product or process), and (2) the utility is specific, substantial, and credible.

2. Review the claims and the supporting written description to determine if the applicant has asserted for the claimed invention any specific and substantial utility that is credible:

(a) If the applicant has asserted that the claimed invention is useful for any particular practical purpose (*i.e.*, it has a "specific and substantial utility") and the assertion would be considered credible by a person of ordinary skill in the art, do not impose a rejection based on lack of utility.

(1) A claimed invention must have a specific and substantial utility. This requirement excludes "throw-away," "insubstantial," or "nonspecific" utilities, such as the use of a complex invention as landfill, as a way of satisfying the utility requirement of 35 U.S.C. 101.

(2) Credibility is assessed from the perspective of one of ordinary skill in the art in view of the disclosure and any other evidence of record (*e.g.*, test data, affidavits or declarations from experts in the art, patents or printed publications) that is probative of the applicant's assertions. An applicant need only provide one credible assertion of specific and substantial utility for each claimed invention to satisfy the utility requirement.

(b) If no assertion of specific and substantial utility for the claimed invention made by the applicant is credible, and the claimed invention does not have a readily apparent well-established utility, reject the claim(s) under § 101 on the grounds that the invention as claimed lacks utility. Also reject the claims under § 112, first paragraph, on the basis that the disclosure fails to teach how to use the invention as claimed. The § 112, first paragraph, rejection imposed in conjunction with a § 101 rejection should incorporate by reference the grounds of the corresponding § 101 rejection.

(c) If the applicant has not asserted any specific and substantial utility for the claimed invention and it does not have a readily apparent well-established utility, impose a rejection under § 101, emphasizing that the applicant has not disclosed a specific and substantial utility for the invention. Also impose a separate rejection under § 112, first paragraph, on the basis that the applicant has not disclosed how to use the invention due to the lack of a specific and substantial utility. The §§ 101 and 112 rejections shift the burden of coming forward with evidence to the applicant to:

(1) Explicitly identify a specific and substantial utility for the claimed invention; and

(2) Provide evidence that one of ordinary skill in the art would have recognized that the identified specific and substantial utility was well established at the time of filing. The examiner should review any subsequently submitted evidence of utility using the criteria outlined above. The examiner should also ensure that there is an adequate nexus between the evidence and the properties of the now claimed subject matter as disclosed in the application as filed. That is, the applicant has the burden to establish a probative relation between the submitted evidence and the originally disclosed properties of the claimed invention.

3. Any rejection based on lack of utility should include a detailed explanation why the claimed invention has no specific and substantial credible utility. Whenever possible, the examiner should provide documentary evidence regardless of publication date (*e.g.*, scientific or technical journals, excerpts from treatises or books, or U.S. or foreign patents) to support the factual basis for the *prima facie* showing of no specific and substantial credible utility. If documentary evidence is not available, the examiner should specifically explain the scientific basis for his or her factual conclusions.

(a) Where the asserted utility is not specific or substantial, a *prima facie* showing must establish that it is more likely than not that a person of ordinary skill in the art would not consider that any utility asserted by the applicant would be specific and substantial. The *prima facie* showing must contain the following elements:

(1) An explanation that clearly sets forth the reasoning used in concluding that the asserted utility for the claimed invention is not both specific and substantial nor well-established;

(2) Support for factual findings relied upon in reaching this conclusion; and

(3) An evaluation of all relevant evidence of record, including utilities taught in the closest prior art.

(b) Where the asserted specific and substantial utility is not credible, a *prima facie* showing of no specific and substantial credible utility must establish that it is more likely than not that a person skilled in the art would not consider credible any specific and substantial utility asserted by the applicant for the claimed invention.

The *prima facie* showing must contain the following elements:

(1) An explanation that clearly sets forth the reasoning used in concluding that the asserted specific and substantial utility is not credible;

(2) Support for factual findings relied upon in reaching this conclusion; and

(3) An evaluation of all relevant evidence of record, including utilities taught in the closest prior art.

(c) Where no specific and substantial utility is disclosed or is well-established, a *prima facie* showing of no specific and substantial utility need only establish that applicant has not asserted a utility and that, on the record before the examiner, there is no known well-established utility.

4. A rejection based on lack of utility should not be maintained if an asserted utility for the claimed invention would be considered specific, substantial, and credible by a person of ordinary skill in the art in view of all evidence of record.

Office personnel are reminded that they must treat as true a statement of fact made by an applicant in relation to

Exhibit 2

SYNOPSIS OF APPLICATION OF THE REVISED INTERIM UTILITY GUIDELINES

It is assumed at this point in the analysis that the specification has been reviewed and an appropriate search of the claimed subject matter has been conducted. It is also assumed that some "utility" is disclosed in the specification or is recognized to be well-established in the art. The examiner should determine whether any asserted utility is specific and substantial, and if so, determine whether such asserted utility is credible. In determining credibility the examiner should consider whether or not there currently are similar or equivalent materials and/or procedures available for achieving that utility. If there are, the utility is credible and no rejection under 35 U.S.C. § 101 should be made.

Guidance for Various Examination Situations

I) a) For method claims that recite more than one utility, if at least one utility is credible, specific, and substantial, a rejection under 35 U.S.C. § 101 should not be made. If any utility in such a claim is not a specific and substantial credible utility, i.e., the claim encompasses at least one utility that does not meet the requirements of 35 U.S.C. §101, the rejection of the claim should be addressed under 35 U.S.C. § 112, first paragraph, scope of enablement.

b) For product claims that do not recite any utilities, disclosure or assertion of one specific, substantial and credible utility meets the criteria of 35 U.S.C. § 101.

II) If no credible, specific, and substantial utility is asserted in the specification and none is well established, a rejection under 35 U.S.C. § 101 would be proper.

III) Cure or prevention - Utilities that constitute curing or preventing a condition are sometimes not credible to one of skill in the art and thus may raise a question under 35 U.S.C. § 101. However, any rejection based on lack of credible utility must be supported by documentary evidence or sound technical reasoning.

IV) Treatment - Since most diseases or conditions can be treated, rejections under 35 U.S.C. § 101 for treatment claims should rarely be made.

V) Vaccines - Since vaccines are regularly prepared to combat various viruses and organisms, vaccines would have a credible utility to one of skill in the art. Thus, vaccines, including those for small pox, should not raise a question under 35 U.S.C. § 101.

VI) Materials to be used for research, or methods of using those materials for research, raise issues of whether the utilities require or constitute carrying out further research to identify or reasonably confirm a "real world" context of use. See, e.g., Brenner v. Manson, 383 U.S. 519, 148 USPQ 689 (Sup. Ct. 1966) wherein a research utility was not considered a "substantial utility."

Definitions

"Credible utility" – Where an applicant has specifically asserted that an invention has a particular utility, that assertion cannot simply be dismissed by Office personnel as being "wrong". Rather, Office personnel must determine if the assertion of utility is credible (i.e., whether the assertion of utility is believable to a person of ordinary skill in the art based on the totality of evidence and reasoning provided). An assertion is credible unless (A) the logic underlying the assertion is seriously flawed, or (B) the facts upon which the assertion is based are inconsistent with the logic underlying the assertion. Credibility as used in this context refers to the reliability of the statement based on the logic and facts that are offered by the applicant to support the assertion of utility. A *credible* utility is assessed from the standpoint of whether a person of ordinary skill in the art would accept that the recited or disclosed invention is currently available for such use. For example, no perpetual motion machines would be considered to be currently available. However, nucleic acids could be used as probes, chromosome markers, or forensic or diagnostic markers. Therefore, the credibility of such an assertion would not be questioned, although such a use might fail the *specific* and *substantial* tests (see below).

"Specific utility" – A utility that is *specific* to the subject matter claimed. This contrasts with a *general* utility that would be applicable to the broad class of the invention. For example, a claim to a polynucleotide whose use is disclosed simply as a "gene probe" or "chromosome marker" would not be considered to be *specific* in the absence of a disclosure of a specific DNA target. Similarly, a general statement of diagnostic utility, such as

diagnosing an unspecified disease, would ordinarily be insufficient absent a disclosure of what condition can be diagnosed.

"Substantial utility" - a utility that defines a "real world" use. Utilities that require or constitute carrying out further research to identify or reasonably confirm a "real world" context of use are not substantial utilities. For example, both a therapeutic method of treating a known or newly discovered disease and an assay method for identifying compounds that themselves have a "substantial utility" define a "real world" context of use. An assay that measures the presence of a material which has a stated correlation to a predisposition to the onset of a particular disease condition would also define a "real world" context of use in identifying potential candidates for preventive measures or further monitoring. On the other hand, the following are examples of situations that require or constitute carrying out further research to identify or reasonably confirm a "real world" context of use and, therefore, do not define "substantial utilities":

A. Basic research such as studying the properties of the claimed product itself or the mechanisms in which the material is involved.

B. A method of treating an unspecified disease or condition. (**Note, this is in contrast to the general rule that treatments of specific diseases or conditions meet the criteria of 35 U.S.C. § 101.**)

C. A method of assaying for or identifying a material that itself has no "specific and/or substantial utility".

D. A method of making a material that itself has no specific, substantial and credible utility.

E. A claim to an intermediate product for use in making a final product that has no specific, substantial and credible utility.

Note that "throw away" utilities do not meet the tests for a *specific* or *substantial* utility. For example, using transgenic mice as snake food is a utility that is neither specific (all mice could function as snake food) nor substantial (using a mouse costing tens of thousands of dollars to produce as snake food is not a "real world" context of use). Similarly, use of any protein as an animal food supplement or a shampoo ingredient are "throw away" utilities that would not pass muster as specific or substantial utilities under 35 U.S.C. §101. This analysis should, of course, be tempered by consideration of the context and nature of the invention. For example, if a transgenic mouse was generated with the specific provision of an enhanced nutrient profile, and disclosed for use as an animal food, then the test for specific and substantial *asserted* utility would be considered to be met.

"Well established utility" - a specific, substantial, and credible utility which is well known, immediately apparent, or implied by the specification's disclosure of the properties of a material, alone or taken with the knowledge of one skilled in the art. "Well established utility" does not encompass any "throw away" utility that one can dream up for an invention or a nonspecific utility that would apply to virtually every member of a general class of materials, such as proteins or DNA. If this were the case, any product or apparatus, including perpetual motion machines, would have a "well established utility" as landfill, an amusement device, a toy, or a paper weight; any carbon containing molecule would have a "well established utility" as a fuel since it can be burned; and any protein would

have well established utility as a protein supplement for animal food. This is not the intention of the statute.

Exhibit 3

EXAMPLE 13: ANTIBODIES TO A SINGLE PROTEIN

Specification:

The specification discloses that a protein designated antigen X has been isolated from HIV and is useful for detection of HIV infections. The specification describes purifying antigen X by gel filtration and discloses its amino acid sequence. Antigen X is further characterized as a 55 kD monomer having no disulfide bonds, with a slightly acidic pI. The specification discusses antibodies which specifically bind to antigen X and asserts that these antibodies can be used in immunoassays to detect HIV. However, there is no working or detailed prophetic example of an antibody that binds to antigen X.

Claim:

Claim 1. An isolated antibody capable of binding to antigen X.

Analysis:

The specification does not describe an actual reduction to practice of an antibody that binds to antigen X by reference to a deposit (e.g., deposit of a hybridoma) or by describing an antibody in structural terms sufficient to show possession. The specification also does not describe the complete structure of an antibody capable of binding antigen X in detailed drawings or through a structural chemical formula. The specification does not describe a partial structure of the claimed antibody. The specification does not describe any physical or chemical properties of the claimed antibody (e.g., molecular weight, association constant).

TECHNICAL NOTE

As evidence, see, e.g., Elvin A. Kabat, STRUCTURAL CONCEPTS IN IMMUNOLOGY AND IMMUNOCHEMISTRY, 2nd Ed. (Holt, Rinehart and Winston 1976), p. 17:

Early studies empirically established that proteins were good antigens when injected into a species other than the one from which they originated. . . . Indeed, it was shown very early in this [20th] century that rabbit serum proteins injected into even as closely related a species as hare would yield antibody (and vice versa). No difficulties were encountered in preparing antibodies to protein antigens from remotely related sources such as bacteria, viruses, and egg, milk, and plant proteins. In most of these studies it sufficed to immunize the animal (an animal receiving injections of an antigen is being immunized) with a solution of the antigen, or preferably, with the protein antigen adsorbed on flocculles of aluminum hydroxide (alum precipitate), since the use of antigens in particulate form had been shown to give a better antibody response.

Exhibit 4

Microbiology (1996), 142, 3135–3145 Printed in Great Britain

Physical mapping of *Mycobacterium bovis* BCG Pasteur reveals differences from the genome map of *Mycobacterium tuberculosis* H37Rv and from *M. bovis*

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A *Dra*I restriction map of the ~4·35 Mb circular chromosome of the vaccine strain *Mycobacterium bovis* BCG Pasteur was constructed by linking all 21 *Dra*I fragments, ranging in size from 6 to 820 kb, using specific clones that spanned the *Dra*I recognition sites as hybridization probes. The positions of 20 known genes were also established. Comparison of the resultant genome map with that of the virulent tubercle bacillus *Mycobacterium tuberculosis* H37Rv revealed extensive global conservation of the genomes of these two members of the *M. tuberculosis* complex. Possible sites of evolutionary rearrangements were localized on the chromosome of *M. bovis* BCG Pasteur by comparing the *Asn*I restriction profile with that of *M. tuberculosis* H37Rv. When selected cosmids from the corresponding areas of the genome of *M. tuberculosis* H37Rv were used as hybridization probes to examine different BCG strains, wild-type *M. bovis* and *M. tuberculosis* H37Rv, a number of deletions up to 10 kb in size, insertions and other polymorphisms were detected. In addition to the known deletions covering the genes for the protein antigens ESAT-6 and mpt64, other genetic loci exhibiting polymorphisms or rearrangements were detected in *M. bovis* BCG Pasteur.

Keywords: *Mycobacterium bovis* BCG Pasteur, tuberculosis, BCG vaccine, genomics

INTRODUCTION

Mycobacterium bovis BCG (Calmette, 1927), an attenuated culture of *M. bovis*, is the world's most widely used vaccine. It has been used to immunize more than two billion people against tuberculosis (Bloom & Fine, 1994), and a very limited number of major side-effects has been reported (Bloom & Murray, 1992). However, protection imparted by BCG against pulmonary tuberculosis is highly variable (Clemens *et al.*, 1983; Fine, 1995) although it is generally accepted that it is efficacious in protecting infants from the severe form of the disease, tuberculous meningitis and miliary tuberculosis.

Since the isolation of the first attenuated culture, from which the strain was never cloned (Calmette, 1927), different variants have emerged during the production of the vaccine in different countries (Bloom & Fine, 1994). Today, the most widely used vaccine strains are BCG Pasteur, BCG Glaxo, BCG Copenhagen and BCG Japan which show morphological, biochemical and immunological differences (Bloom & Fine, 1994; Lagranderie *et al.*, 1996). Although it is recommended that BCG be used in areas where tuberculosis is endemic, a better vaccine is required for the elimination of the disease. Analysis of the BCG genome will provide us with important information that will help in constructing BCG strains with improved immunological properties and may lead to the rational attenuation of *Mycobacterium tuberculosis*.

The construction of genomic maps of nearly 100 bacterial chromosomes has led to improved understanding of their general organization and evolution (Cole & Saint-Girons, 1994; Fonstein & Haselkorn, 1995; Krawiec & Riley, 1990). Data about genome size, architecture and topology can be very rapidly generated by a combination of physical and genetic analysis, and often enable regions of interest

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Table 1. Different members of the *M. tuberculosis* complex analysed in this study

Strain	Source*
M. tuberculosis H37Rv	GMB
M. tuberculosis H37Ra	GMB
M. boris ATCC 19210	Institut Pasteur Collection (1400020001)
M. bovis BCG Pasteur	LBCG
M. boris BCG Denmark	LBCG
M. boris BCG Glaxo	LBCG
M. bovis BCG Japan	LBCG
M. bovis BCG Moreau	LBCG

*GMB, Unité de Génétique Moléculaire Bactérienne; LBCG, Laboratoire du BCG, Institut Pasteur, Paris, France.

to be identified and analysed in detail. Information is lacking about the genomic organization of *M. bovis* BCG and its progenitor, but might be useful for the molecular dissection of the genetic basis of the pathogenicity of the tubercle bacillus. Extending this approach to the different BCG substrains could explain some of the phenotypic and immunological differences. Here, the construction of a physical map of the genome of *M. bovis* BCG Pasteur is described, and the results of global comparison with the genome map of *M. tuberculosis* H37Rv, and local analysis of selected polymorphic regions in four other BCG strains and in *M. bovis*, are presented. The availablity of a high resolution, integrated genomic map of *M. tuberculosis* H37Rv (Philipp *et al.*, 1996) facilitated this task and led to the localization of areas of possible rearrangements.

METHODS

Preparation of agarose-embedded chromosomal DNA. BCG strains (Table 1) were grown on Sauton medium, and *M. tuberculosis* H37Rv and H37Ra were cultured in Dubos medium (Pasteur Diagnostics) supplemented with oleic acid/albumin/dextrose/catalase (OADC; Difco), and incubated for 10–12 d at 37 °C. D-Cycloserine (1 mg ml^{-1}) was added and incubation continued for a further 24 h prior to harvesting of cells at 4000 r.p.m. in a Sorvall RC-5B centrifuge. The cells were resuspended in 1 × TE buffer (10 mM HCl, 1 mM EDTA, pH 7·6), enclosed in 1% (w/v) low melting point agarose (BRL) and further processed to release intact genomic DNA as described previously (Philipp *et al.*, 1996).

PFGE and conventional gel electrophoresis. Chromosomal DNA from the different strains was digested to completion after incubating agarose plugs overnight in the appropriate buffers containing 30 U *Dra*I (Gibco BRL) in the presence of 0·1% Triton X-100 (Serva) and 30 U *Asn*I (New England Biolabs) or 30 U *Eco*RI (Boehringer Mannheim), then washed in 1 × TE buffer before loading. In some experiments *Pme*I, or the intron-encoded endonucleases I-*Ceu*I, I-*Ppo*I and I-*Tlu*I (all from New England Biolabs) or I-*Sce*I (Boehringer Mannheim) were used either alone or in conjunction with *Dra*I.

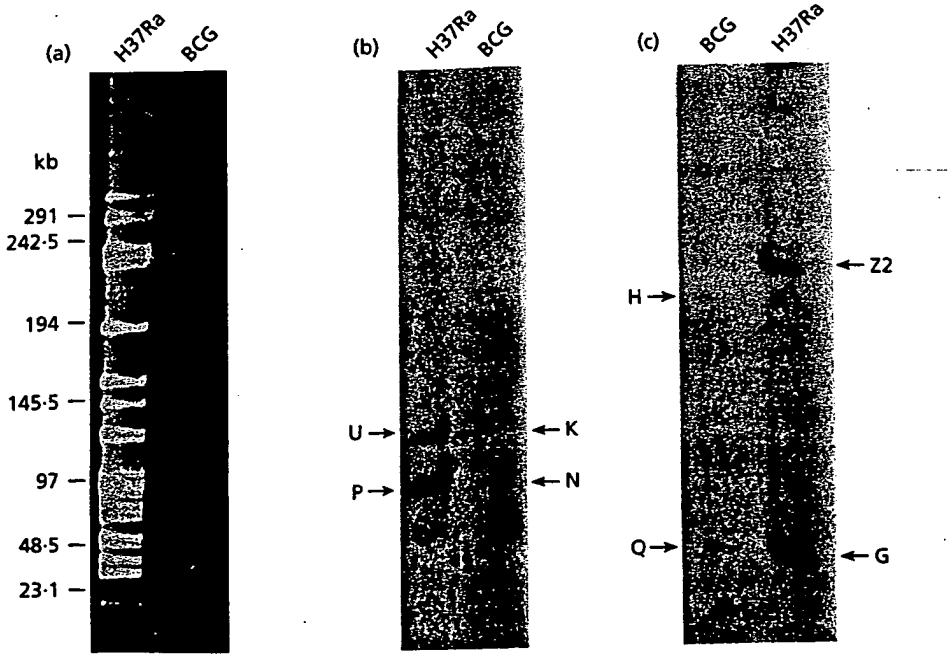

Fig. 1. Comparison of the *Dra*I profiles and linking analysis of *M. bovis* BCG Pasteur and *M. tuberculosis* H37Ra. (a) Ethidium-bromide-stained pulsed field gel on which *Dra*I restriction fragments of *M. bovis* BCG Pasteur and *M. tuberculosis* H37Ra have been separated. (b), (c) Corresponding Southern blot with two linking clones of *M. tuberculosis* H37Rv used as hybridization probes: probe Y2 that links fragments U and P or K and N (b) and probe T225 that links fragments Q and H or Z2 and G (c) in *M. tuberculosis* H37Ra or *M. bovis* BCG Pasteur, respectively. The positions of key size markers are indicated.

Table 2. Restriction fragments of the chromosome of *M. bovis* BCG Pasteur and comparison with those of *M. tuberculosis* H37Rv

DraI				*AseI*			
BCG		H37Rv		BCG		H37Rv	
Fragment	Size (kb)	Size (kb)	Fragment	Fragment	Size (kb)	Size (kb)	Fragment
A	820			A	700	700	V
B	700			B	340	340	U
		580	Z7				
C	480	475	Z6	C	280		
						240	T
D	370			D	235	235	S
		300	Z5				
E	250	260	Z4	E	190		
						180	R
F	230	230	Z3	F	170	170	Q
G	230	230	Z2	G	155	155	P
						137	O3
						135	O2
H	210	220	Z1	H3	133	133	O1
I	210			H2	132		
		190	Y1/Y2				
		165	X1				
J	160	160	W	H1	131		
		140	V			130	N
K	125	125	U	I3	125	125	M1/M2
		120	T				
L	100			I1/2	122		
		95	S				
M	87	87	R	J	115	115	L
N1	87	87	Q1	K	105	105	K
N2	87	87	Q2	L1/2	100	100	J1/J2
		82	P				
		78	O				
		72	N			78	I
O	65	65	M	M	75		
P	60	60	L	N	73	73	H
		47	K/J				
		40	I				
		33	H				
Q	30	30	G	O7	65	65	G8
R	26	26	F	O6	63	63	G5/6/7
		23	E				
		22	D				
S	13	13	C	O5	61	61	G4
		7	B				
T	6	6	A	O4	59	59	G2/3
		2·2	A1				
				O3	57	57	G1
				O2	55		
				O1	53		
				P1/2	50	50	F1/2
						47	E4
						46	E3
				Q1/2/3	45	42	E2
						44	E1
				R	37	37	D
				S	35		
				T	33	33	C4
				U	32		
						31	C3
				V	30	30	C1/2
				W	25		
				X1/2	22		
				Y5	18	18	B6
				Y3/4	15	15	B4/5
						13	B3
				Y2	11	11	B2
				Y1	9	9	B1
				Z4	4·5	4·5	A4
				Z3	4	4	A3
				Z2	3·5	3·5	A2
				Z1	3	3	A1
Total	4346	4394			4370	4407	

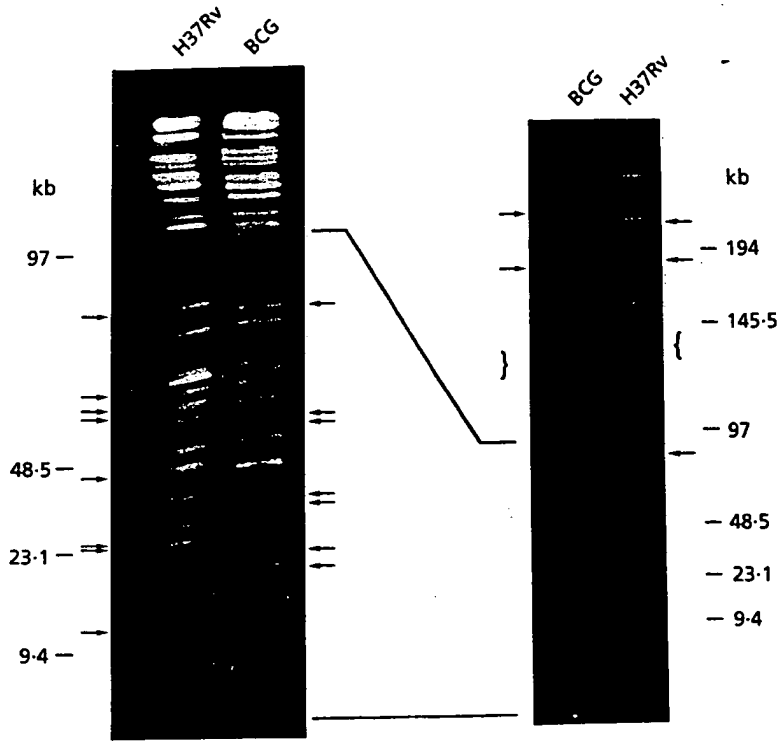

Fig. 2. Ethidium-bromide-stained gel of an *Asn*I digestion of the chromosomes of *M. bovis* BCG Pasteur and *M. tuberculosis* H37Rv after separation by PFGE. The left and right panels correspond to gels on which fragments up 100 kb and up to 700 kb, respectively, were separated. The arrows indicate the principal differences in the restriction patterns and the brackets the compressed zone.

*Dra*I and *Asn*I restriction fragments were separated on 1·2% agarose gels using a CHEF II Pulsaphor apparatus (Pharmacia) with pulse times of 3 s for 20 h for fragments up to 100 kb, 10 s for 20 h for fragments up to 300 kb, and 20 s for 20 h for the largest fragments, up to 1 Mb. Electrophoretic runs were carried out at 280 V and 10 °C. DNA was subsequently Southern-blotted, transferred (Philipp *et al.*, 1996; Sambrook *et al.*, 1989) to Hybond-C or Hybond-N membranes (Amersham) and then used for hybridization experiments. *Eco*RI restriction fragments were resolved on 0·7% agarose gels using standard conditions and then processed for hybridization experiments as described above.

Library construction and screening for linking clones. Chromosomal DNA (2 µg) was partially digested with 0·07 U *Sau*3AI (Boehringer Mannheim) and size-fractionated on a 1% agarose gel. Fragments of 1–3 kb in size were purified by the Geneclean method (Bio101), then ligated into the dephosphorylated kanamycin-resistant vector pUC19GGK and transformed into *Escherichia coli* DH5α (Sambrook *et al.*, 1989). Plasmid DNA was subjected to digestion with *Dra*I and positive clones sorted into groups for hybridization experiments. In parallel, known *Dra*I linking clones from the pYUB18 cosmid library of *M. tuberculosis* H37Rv were used in hybridization experiments together with probes for well-characterized genes (Philipp & Cole, 1995; Philipp *et al.*, 1996).

Hybridization. Cosmid or plasmid DNA (100 ng) was labelled with [α-^{32}P]dCTP (ICN) by nick translation and linear molecules were labelled by random priming with the multiprime kit (Amersham). Labelled probes were purified by low speed centrifugation on P10 micro-columns (BioRad) then transferred to a solution containing 5 × SSC (1 × SSC is 0·15 M sodium chloride, 0·015 M sodium citrate) and 50% (w/v) formamide (Philipp *et al.*, 1996; Sambrook *et al.*, 1989). Filters were washed after hybridization at 37 °C overnight in a final concentration of 0·1 × SSC at 65 °C or at room temperature as required.

RESULTS

The mapping strategy

In a previous study, a detailed physical map of the genome of the tubercle bacillus *M. tuberculosis* H37Rv was constructed using a combined approach involving hybridization analysis with linking clones to join macro-restriction fragments obtained after digestion of genomic DNA with *Asn*I or *Dra*I, followed by verification and extension by two-dimensional gel electrophoresis of reciprocal *Asn*I and *Dra*I digests (Philipp *et al.*, 1996). As *M. bovis* BCG is highly related to *M. tuberculosis* it seemed probable that the *M. tuberculosis* linking probes, as well as the unique coding sequences, would be useful for mapping the BCG genome. However, since it was also conceivable that some of the rare restriction sites might be confined to one or other species, it was decided that a second linking library should be constructed from *M. bovis* BCG Pasteur.

PFGE

Since the genomes of species from the *M. tuberculosis* complex have a high G+C content, a series of restriction enzymes recognizing A+T-rich sites was tested to identify the most appropriate ones and, in parallel, a battery of intron-encoded endonucleases was evaluated. None of the latter enzymes, including the immensely versatile I-*Ceu*I which cleaves in the *rrl* gene, encoding the 23S rRNA of most bacteria and lower eukaryotes (Liu *et al.*, 1993; Marshall & Lemieux, 1992), showed evidence of cleavage in single or double digestions. Subsequent inspection of the sequence of the *M. tuberculosis rrl* gene (Bergh & Cole, 1994) revealed a single base deletion in the sequence generally recognized by I-*Ceu*I (Marshall & Lemieux, 1992).

As found previously with *M. tuberculosis* H37Rv (Cole & Smith, 1994; Philipp *et al.*, 1996), digestion of the chromosome of *M. bovis* BCG with *Dra*I (TTTAAA) yielded the lowest number of fragments, 21, ranging in size between 6 and 820 kb (Fig. 1; Table 2). Thirteen of these fragments displayed identical electrophoretic mobilities to *Dra*I fragments obtained from the chromosome of *M. tuberculosis* indicating that they were probably equivalent (Table 2). *Pme*I which recognizes an extended *Dra*I site (GTTTAAAC) was found to cleave the BCG genome at a single site (data not shown) and the genome of *M. tuberculosis* at two sites.

Digestion with *Asn*I (ATTAAT) generated 48 fragments from BCG Pasteur and 47 from *M. tuberculosis* (Fig. 2). Many of them (33 out of 48) were identical in size and the sum of the resultant fragment sizes indicated that the genome consisted of ~4370 kb. A similar value was obtained by summing *Dra*I fragment sizes (~4346 kb, Table 2) but in both cases the total BCG genome size was ~40 kb smaller than that of *M. tuberculosis*. Because the migration of some of the fragments resulted in the creation of two zones of compression (around 87 kb, 3 fragments; and between 210 and 250 kb, 5 fragments with *Dra*I, and in the 15–75 kb range with *Asn*I) it was necessary to use several different running conditions for optimal separation. Typical gels giving optimal resolution of *Asn*I fragments in the 100–700 kb and 10–100 kb ranges are shown in Fig. 2. Despite these modifications, the *Dra*I fragments around 87 kb could not be separated to completion, so their locations on the BCG map, and those of the fragments I/H and F/G, which are located in the second compression, were inferred by comparison with the map of *M. tuberculosis* (Philipp *et al.*, 1996) and confirmed by hybridization analysis (see below).

Table 3. Linking and localization analysis of *M. tuberculosis* H37Rv and *M. bovis* BCG Pasteur chromosomes using various probes

Probe	H37Rv		BCG
	*Dra*I	*Asn*I	*Dra*I
T4	P/Q1	Q	M/B
T57	Z5/S	R	B
T67	J	C2	D
T143	Z7	U	A
T145	U	V	K
T225	Z2/G	H	H/Q
T288	M+D	L	O/P
T352	Z6/F	V	C/R
T670	Z1/E	C4	G/L
T876	S/Q2	G2	B
T715	Z7	U	A
T907	C/A/L	O3	F/S
Y2	U/P	V	K/N
Y28	B/I/Y1	M2	E
Y63	U/P	V	K/N
Y96	Z3/O	O3	I/N2
Y98	D/K	E1/L	O
Y123	H/K	E1/O1	F/O
Y136	Z6	E4	C
Y153	Y1/Z4	J1	A/E
Y185	Z1/X	V	G/J
Y202	Q1/P	Q	M/B
Y216	Y1	P	E
Y342	L	I	F
Y346	Z6	C1+E4	C
Y363	Y1	P	E
Y414	A/V/G	H	T/D/Q
Y428	J/M	L	D/P
Y489	Z6	S	C
pGG34	NT	NT	A/E
pGG25	NT	NT	C/R
pGG11	NT	NT	N/J
pGG175	NT	NT	R/K
pGG174	NT	NT	N2/I
pGG224	NT	NT	S/N2
pGG221	NT	NT	J/G
pGG256	NT	NT	A/L
Mt_AsnI_Q	P/Q1	Q	H/M

NT, Not tested.

Construction of a BCG linking library

Two libraries were constructed in different vectors to isolate BCG DNA harbouring *Dra*I restriction sites. Clones with inserts bearing a *Dra*I site were identified, sorted into groups and then used in hybridization experiments on total genomic *Dra*I digests to find the naturally-existing links between the *Dra*I macrorestriction fragments. Screening of 150 clones from these libraries resulted in the identification of 14 independent *Dra*I linking clones, the pGG series (Table 3), which were subsequently used in linking analysis.

The genome map

To determine the order and orientation of the 21 *Dra*I restriction fragments, suitable Southern blots of BCG DNA were hybridized with a panel of 7 BCG and 19 *M.*

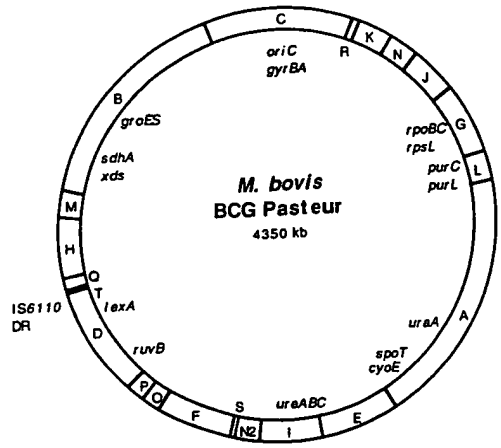

Fig. 3. The circular *Dra*I restriction map of the chromosome of *M. bovis* BCG Pasteur. The positions of some genetic markers (see Table 4) and of the insertion sequence IS*6110* are shown. The position of the direct repeat region that harbours the sole copy of IS*6110* in *M. bovis*, BCG strains and some clinical isolates of *M. tuberculosis* is indicated by 'DR'.

Table 4. Identity and source of known genetic markers mapped in *M. bovis* BCG Pasteur

Gene	Description	Source/ reference*
cyoE	Cytochrome *o* ubiquinol oxidase	Mt, GMB
groES	HSP-12; chaperone	Mt, T. Shinnick
groEL-2	HSP-60 homologue	Mt, GMB
gsa	Glutamate-1-semialdehyde 2,1-aminomutase	Mt, GMB
gyrAB	Gyrase, A and B subunits	Mt, H. E. Takiff
lexA	SOS regulator	Mt, GMB
oriC	Origin of replication	Mt, H. E. Takiff
purC	Purine synthesis	Mt, UGM
purL	Purine synthesis	Mt, UGM
rpsL	Ribosomal protein S12	Mt, GMB
rpoBC	RNA polymerase, β and β' subunits	Mt, GMB
ruvB	DNA helicase	Mt, GMB
sdhA	Succinate dehydrogenase	Mt, GMB
sodA	Superoxide dismutase	Mt X16453
spoT	Pyrophosphohydrolase	Mt, GMB
uraA	Orotidine-5'-phosphate decarboxylase	Mb, R. A. Young
ureABC	Urease, subunits A, B and C	Mt, UGM
xds	Unique sequence for detection of *M. tuberculosis* complex	Mt, GMB

*Mt, *M. tuberculosis*; Mb, *M. bovis*; GMB, Unité de Génétique Moléculaire Bactérienne and UGM, Unité de Génétique Mycobactérienne, Institut Pasteur, Paris, France; T. Shinnick, Centers For Disease Control, Atlanta, GA, USA; H. E. Takiff, Instituto Venezolano de Investigaciones Cientificas, Caracas, Venezuela; R. A. Young, Whitehead Institute for Biomedical Research, Cambridge, MA, USA.

tuberculosis linking clones (Table 3). A representative linking analysis is shown in Fig. 1. It can be seen that identically sized fragments of 125 and 80 kb (Table 2) in the chromosomes of *M. bovis* BCG Pasteur (K and N2, respectively) and *M. tuberculosis* H37Ra (U and P, respectively) were contiguous (Fig. 1b). By contrast, hybridization with probe T225 (Fig. 1c) shows that, while one of two adjacent fragments (30 kb; Q in BCG, G in *M. tuberculosis*; Table 2) was the same size in both mycobacteria, the other was about 20 kb smaller in the genome of BCG (H in BCG, Z2 in *M. tuberculosis*; Table 2). Linkage of fragments H and M was obtained by using *Asn*I fragment Q from *M. tuberculosis* as a probe. In this way all 21 *Dra*I restriction fragments were linked, thereby revealing a single circular chromosome (Fig. 3). Additional proof, albeit indirect, of the circularity of the chromosome of *M. bovis* BCG was obtained by using optimized PFGE running conditions for resolving linear megabase-sized DNA molecules since undigested circular genomic DNA did not enter the gels. Under these conditions, the linear chromosome of *Agrobacterium tumefaciens* was resolved (Allardet *et al.*, 1993; Fonstein & Haselkorn, 1995) whereas that of BCG Pasteur remained at the origin.

The above linking and restriction analysis suggested that the chromosomes of *M. tubeculosis* H37Rv and *M. bovis* BCG were indeed very similar. To determine whether the positions of known genes were also conserved (Philipp *et al.*, 1996), probes corresponding to 18 different genetic markers (see Table 4) were used in hybridization experiments with *Dra*I and *Asn*I fragments resolved by PFGE. The locations of all markers tested were compared with those of their homologues in the genome of *M. tuberculosis*. The DNA gyrase genes *gyrAB*, and *oriC*, the chromosomal origin of replication of *M. tuberculosis*, hybridized to *Asn*I and *Dra*I fragments of the same size in the chromosomes of both species. This was also the case for the genes *rpsL*, *rpoB* and *ureC* (Philipp & Cole, 1995). The results of this analysis, summarized in Figs 3 and 4, revealed extensive genome-wide conservation.

Detailed molecular comparison with *M. tuberculosis* H37Rv

The linking clone analysis strongly suggested that all 21 *Dra*I cleavage sites were conserved in both *M. bovis* BCG and *M. tuberculosis* H37Rv although the fragments varied in size and number (Table 2). This variation can be explained by differences in the copy number of the insertion sequence IS*6110*, which carries a *Dra*I site (Thierry *et al.*, 1990a, b), as a single copy is present in *M. bovis* whilst 16 copies have been mapped in *M. tuberculosis* (Hermans *et al.*, 1991; Philipp *et al.*, 1996). Although extensive conservation of *Asn*I sites and fragment lengths were detected (at least 33 out of 47 were common), a number of fragments appeared to differ significantly (see Table 2). In BCG Pasteur, most of these *Asn*I fragments corresponded to regions of the chromosome of *M. tuberculosis* H37Rv where repetitive sequences are present

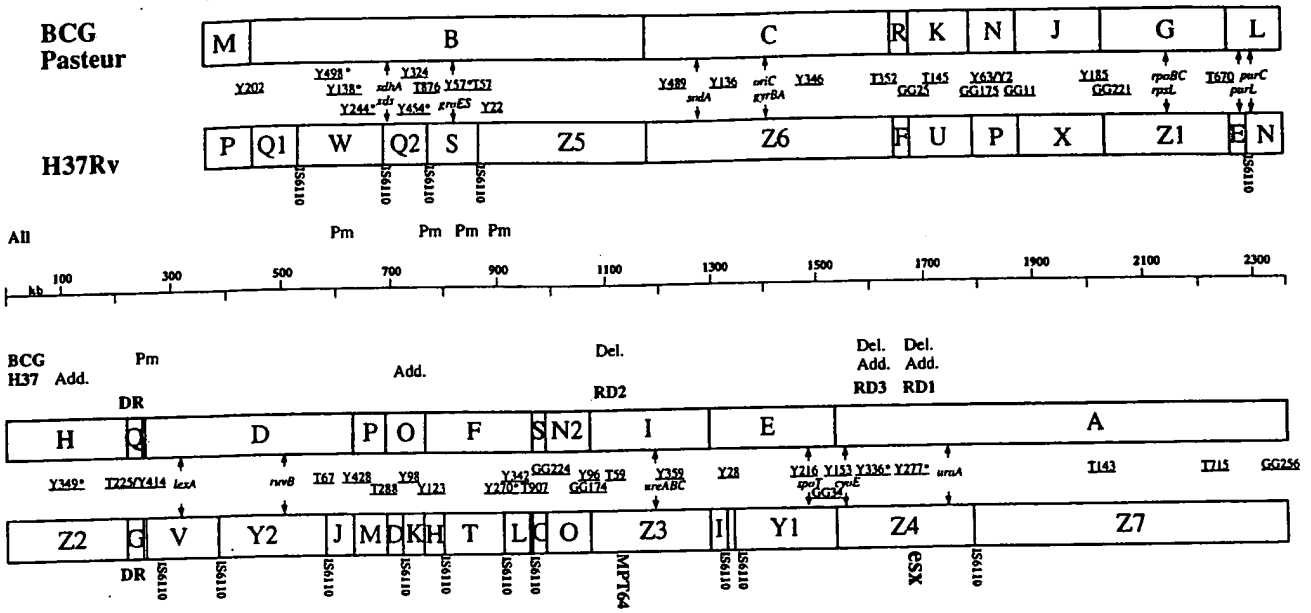

Fig. 4. Schematic presentation of the chromosomes of *M. bovis* BCG Pasteur and *M. tuberculosis* H37Rv by alignment of their *Dra*I fragments. The approximate locations of mapped genes, linking clones and cosmids used as probes for detection of variable regions are indicated. Cosmids that were only hybridized to blots of *Eco*RI digests are denoted by an asterisk. Variable regions are highlighted in the central portion: Pm, polymorphisms; Add., additional DNA; Del., deletions. The positions of the regions deleted from the chromosome of BCG Connaught, RD1–RD3 (Mahairas *et al.*, 1996), are indicated. Other details are as in the legend to Fig. 3.

(Fig. 4) and this suggested that these loci may be readily susceptible to rearrangements, insertions and deletions.

To test this possibility, 15 cosmids were selected from the ordered library of H37Rv clones for use as hybridization probes to try and detect differences in the *Eco*RI restriction patterns of the respective genomes. For control and comparative purposes, DNA from *M. bovis* and BCG substrains Copenhagen, Glaxo, Moreau and Tokyo was examined and the combined results of this study are summarized in Table 5 and Fig. 4. Eleven of the 15 cosmids used in hybridization detected genomic differences due to insertions, deletions and restriction fragment length polymorphisms in the comparative genomic analysis. Certain probes detected fragments in *M. tuberculosis* that were also present in *M. bovis* but were apparently missing from the genomes of some, or all, of the various BCG strains (Y57, Y277, T59 and Y366) while others only hybridized to sequences that were present in *M. tuberculosis*, and not in *M. bovis* or BCG (Y22, Y98, Y138, Y277, Y324, Y349 and Y366). In one case (Y57; Fig. 5b), a polymorphism was detected only in *M. bovis*, whereas with probe Y324 the pattern obtained with all BCG strains resembled more closely that of *M. tuberculosis* than *M. bovis* (Fig. 5c). The region covered by probe Y277 is of particular interest as it corresponds, in part, to the locus encoding ESAT-6. The corresponding gene, *esx*, is known to be present in *M. tuberculosis* and *M. bovis* on a large (> 15 kb) *Eco*RI fragment and is missing from BCG (Sorensen *et al.*, 1995) as can be seen in (Fig. 5a). Strikingly, this region of the chromosome of *M. tuberculosis* H37Rv also contained three other *Eco*RI fragments that do not appear to have counterparts in *M. bovis* or BCG (Table 5). Similar results were obtained with probe Y366 which detected two additional *Eco*RI fragments in *M. tuberculosis* H37Rv compared to *M. bovis*, and indicated that a deletion had occurred in the corresponding region of the chromosome of BCG Pasteur as at least two fragments seemed to be missing (Fig. 5e, Table 5).

DISCUSSION

The principle objective of this work was to construct a physical map of the chromosome of the classical vaccine strain *M. bovis* BCG Pasteur and hence to obtain general insight into its genomic organization. BCG has served as a safe live vaccine for over 70 years (Bloom & Fine, 1994), and the comparative dissection of its genome could help to identify regions that differ extensively from the corresponding segments of the chromosomes of the pathogens *M. bovis* and *M. tuberculosis*. The recent completion of an integrated map of the circular chromosome of the tubercle bacillus *M. tuberculosis* H37Rv, and the tools generated therein (Philipp *et al.*, 1996), considerably facilitated this task in the absence of the parental *M. bovis* strain originally isolated by Calmette, which was lost during the war (Calmette, 1927).

Comparison of the genome maps of *M. bovis* BCG Pasteur and the virulent reference strain *M. tuberculosis* H37Rv revealed that there was extensive conservation of genes,

Table 5. Comparison of patterns after hybridization of selected cosmids on complete *Eco*RI digestions of the chromosomes of *M. tuberculosis* H37Rv, wild-type *M. bovis* and different *M. bovis* BCG strains

Probe	Mycobacterial species	Approximate fragment size (kb)
T59	*M. tuberculosis*	8, 7, 6, 4, 1·5
	M. bovis	ND
	BCG Pasteur	8, 7, 6, 4, 1·5
Y22	*M. tuberculosis*	15, 12, 7, 5·5, 4·5, 3
	M. bovis	15, 12, 7, 5·5, 4·5, –
	BCG Pasteur	15, 12, 7, 5·5, 4·5, –
	BCG Moreau	15, 12, –, 5·5, 4·5, –
Y57	*M. tuberculosis*	15, 8, 5, –, 3·5, 2·5, 1·8, 1·5, 1·3, 1
	M. bovis	15, 8, 5, 4, –, 2·5, 1·8, 1·5, 1·3, 1
	BCG Pasteur	15, 8, 5, –, –, 2·5, 1·8, 1·5, 1·3, 1
Y98	*M. tuberculosis*	12, 9, 6, 5·5, 4·5, 3·8, 3·3, 3, 2·5, 2, 1·5, 0·8, 0·6, 0·5, 0·3
	M. bovis	12, 9, 6, 5·5, 4·5, 3·8, –, 3, 2·5, 2, 1·5, 0·8, 0·6, 0·5, –
	BCG Pasteur	12, 9, 6, 5·5, 4·5, 3·8, –, 3, 2·5, 2, 1·5, 0·8, 0·6, 0·5, –
Y138/Y498	*M. tuberculosis*	> 15, 15, –, 5·5, 4·5, 2·7, 1
	M. bovis	> 15, 15, –, 5·5, 4·5, 2·7, 1
	BCG Pasteur	> 15, 15, –, 5·5, 4·5, 2·7, 1
	BCG Glaxo	> 15, 15, 6, 5·5, 4·5, 2·7, 1
	BCG Copenhagen	> 15, 15, 6, 5·5, 4·5, 2·7, 1
Y277	*M. tuberculosis*	> 15, 9, 5·5, 4, 3·5, 3, 2·5, 2·3, 0·75, 0·5
	M. bovis	> 15, 9, –, –, 3·5, 3, 2·5, 2·3, 0·75, 0·5
	BCG Pasteur	–, 9, –, –, 3·5, 3, 2·5, 2·3, 0·75, 0·5
Y324	*M. tuberculosis*	9·5, 8·5, 6, 5·1, 4·5, 4, 3·5, 2·9, –, 2·1, 1·3, 1·2, 0·8
	M. bovis	9·5, 8·5, 6, –, 4·5, –, 3·5, 2·9, 2·2, 2·1, 1·3, 1·2, 0·8
	BCG Pasteur	9·5, 8·5, 6, 5·1, 4·5,-, 3·5, 2·9, 2·2, 2·1, 1·3, 1·2, 0·8
Y349	*M. tuberculosis*	> 8, 6, 5, 4, 3·5, 2·7, 2, 0·7, 0·5
	M. bovis	> 8, 6, –, 4, 3·5, 2·7, 2, 0·7, 0·5
	BCG Pasteur	> 8, 6, –, 4, 3·5, 2·7, 2, 0·7, 0·5
Y366	*M. tuberculosis*	> 11, 11, 5·5, 4, 3·7, 3·2, 3·1, –, 2·1, 1·7, 0·6
	M. bovis	> 13, 9, –, 4, 3·7, 3·2, 3·1, 2·2, 2·1, –, 0·6
	BCG Pasteur	10, –, –, 4, 3·7, 3·2, 3·1, –, 2·1, –, 0·6
Y414	*M. tuberculosis*	12·5, 12, 6, 5, 3·5, 2·5, 1·8, 1·1, 0·7, 0·5
	M. bovis	12·5, 12, 6, 5, 3·5, 2·5, 1·8, 1·1, 0·7, 0·5
	BCG Pasteur	12·5, 12, –, 5, 3·5, 2·5, 1·8, 1·1, 0·7, 0·5
	Other BCGs	12·5, 12, 6, 5, 3·5, 2·5, 1·8, 1·1, 0·7, 0·5
Y454	*M. tuberculosis*	6, 5, 4, 3·5, 3·2
	M. bovis	ND
	BCG Pasteur	6, 5, 4

ND, Not determined.

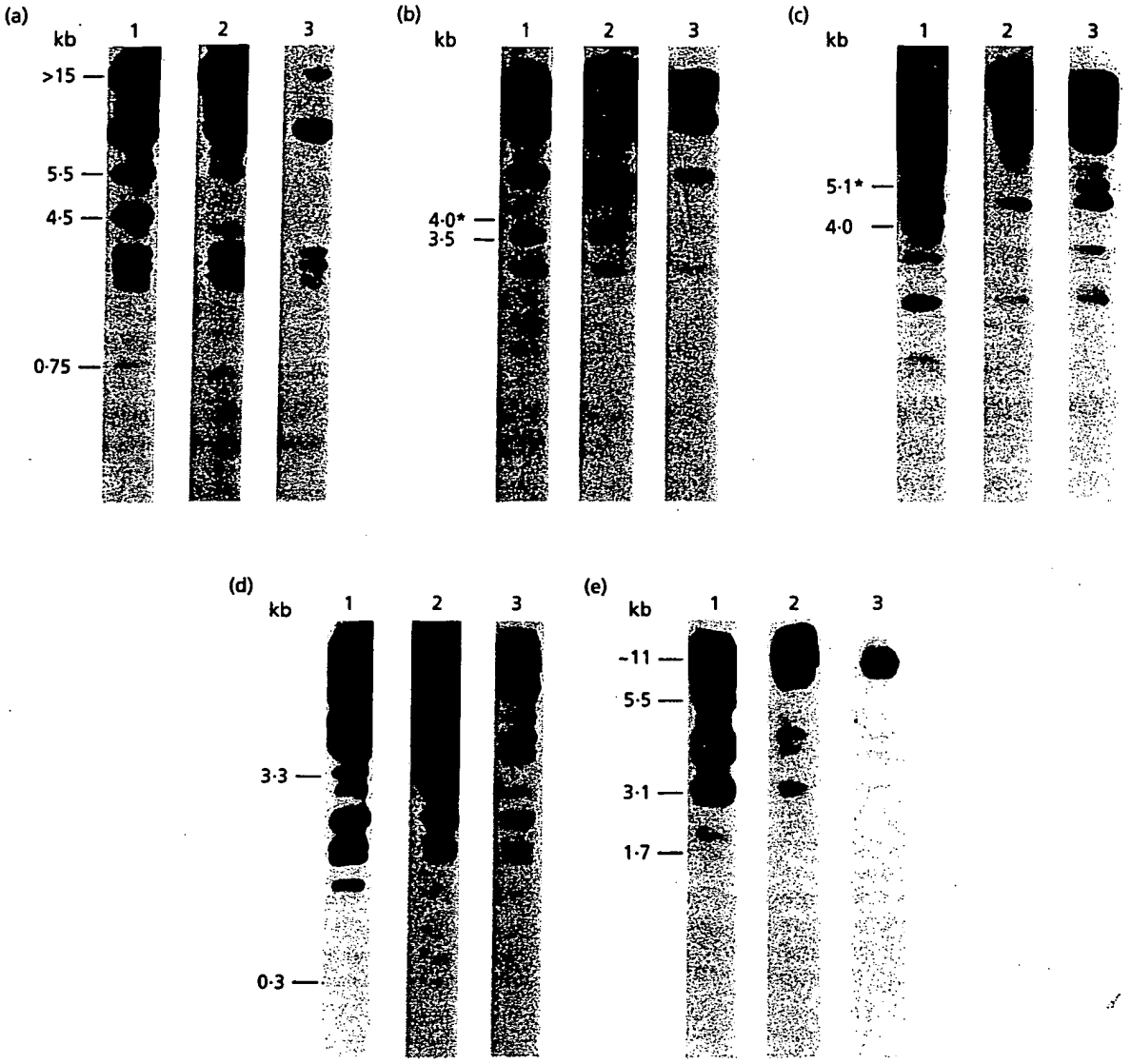

Fig. 5. Analysis of variable regions of the chromosome of different *M. bovis* BCG strains by hybridization with selected cosmids. Samples were complete *Eco*RI digests from *M. tuberculosis* H37Rv (lane 1), *M. bovis* (lane 2) and *M. bovis* BCG Pasteur (lane 3). Hybridization was performed with probes prepared from cosmids Y277(a), Y57 (b), Y324 (c), Y98 (d) and Y366 (e). The sizes of fragments that differ in *M. tuberculosis* and in *M. bovis* are labelled without and with an asterisk, respectively. Owing to the use of nick-translated cosmids as probes some of the smaller fragments do not show up well in these autoradiograms but were visible on longer exposure. The results of other hybridizations are summarized in Table 5 and Fig. 4.

marker order, restriction sites and fragment sizes, indicating that the two genomes were essentially colinear (Figs 3, 4; Tables 2, 3, 5). The least divergent segment of the genome is a 1500 kb stretch centred around the *oriC* locus (Fig. 4). The finding of global conservation is consistent with the fact that comparison of the sequences of genes from these two organisms and their strains reveals an identity of ~99% (Kapur *et al.*, 1994). Nevertheless, comparison of the restriction patterns, the *Asn*I fragment sizes and the respective genome maps led to the identification of regions that appeared to differ in their local organization. In several cases where differences were suspected they were confirmed by hybridization analysis using cosmids carrying segments of the *M. tuberculosis* chromosome as probes. The variable regions could be grouped into three classes: the first, where *M. tuberculosis* contains DNA that is not present in *M. bovis* or BCG (Fig. 5a, d); the second, in which the arrangement in *M. tuberculosis* and *M. bovis* appears to be the same but differs from that of BCG (Fig. 5a, c); the third where *M. tuberculosis* and BCG are similar but *M. bovis* displays a restriction fragment length polymorphism (Fig. 5c).

There are two likely explanations for the first class of polymorphisms; either they correspond to unique genomic sequences that are confined to H37Rv and possibly other isolates of *M. tuberculosis*, or they represent an insertion event such as acquisition of an IS element or expansion of a repetitive sequence. The latter explanation may be correct in some cases but it is not true of all. For instance, two and three additional *Eco*RI fragments were detected with probes Y98 and Y277, respectively (Fig. 5a, d), whereas only one polymorphic fragment would be expected if any of the known insertion sequences in *M. tuberculosis* had transposed as none of these contains a site for *Eco*RI. A number of important phenotypic differences between *M. bovis*, or BCG, and *M. tuberculosis* are known (Heifets & Good, 1994). Neither *M. bovis* or BCG is capable of respiration with nitrate or able to produce niacin, unlike *M. tuberculosis*. Both *M. bovis* and BCG are resistant to pyrazinamide but susceptible to 2-thiophene-carboxylic acid hydrazide (TCH) whereas *M. tuberculosis* displays the opposite phenotypes. While *M. bovis* is able to infect humans efficiently, *M. tuberculosis* is not pathogenic for bovines. It is conceivable that some of these discrepant phenotypes may have their origins in the divergent genomic areas described here. The availability of well-characterized shuttle cosmid clones (Philipp *et al.*, 1996), will allow this hypothesis to be tested.

The second class of polymorphisms corresponds to three loci that appear to have undergone deletion events during the isolation of the original BCG Pasteur strain and subsequent evolution of the various BCG substrains. In two instances, regions which differ between the two mapped mycobacterial genomes and appear to have contracted in BCG Pasteur are known to harbour the genes for *M. tuberculosis* protein antigens, MPT64 and ESAT-6. Other workers have demonstrated that these coding sequences are missing from some (MPT64) or all (ESAT-6) BCG strains (Harboe *et al.*, 1996; Li *et al.*, 1993; Sorensen *et al.*, 1995) but present in virulent tubercle bacilli. Recently, by means of a genomic subtraction approach involving *M. bovis* and BCG Connaught, Mahairas *et al.* (1996) identified and extensively characterized three regions termed RD1–RD3, which had been deleted from the chromosome of BCG Connaught. Two of the RDs encoded the MPT64 and ESAT-6 antigens while RD1 contained a regulatory locus that influences production of several proteins. The findings of these workers are consistent with the mapping data described here and the positions of the RD regions are indicated in Fig. 4. It is of interest that these regions susceptible to deletions are located towards the putative mycobacterial replication terminus as it is known from work with other bacteria that the terminus is often a site of genetic rearrangements (Krawiec & Riley, 1990). Furthermore, RD1 and RD3 are situated within the same 100 kb mapping interval (Fig. 4).

In a recent authoritative study (Lagranderie *et al.*, 1996), it was convincingly demonstrated that different BCG strains could be classified in terms of their growth rates and of the immune responses that they induced in mice. Two of the strains examined, the Prague and Japanese BCG strains, could not confer resistance against a second inoculation with various recombinant BCG strains whereas immunization with the Glaxo, Pasteur or Russian strains could. The preliminary results presented in Table 5 indicate that polymorphisms exist between the various BCG substrains and detailed analysis of the genomic organization may well shed further light on the genetic basis of this immunovariability by highlighting polymorphic segments of the chromosome. The genome map of BCG Pasteur established in the present work will thus be of great value for the interpretation of further comparative studies.

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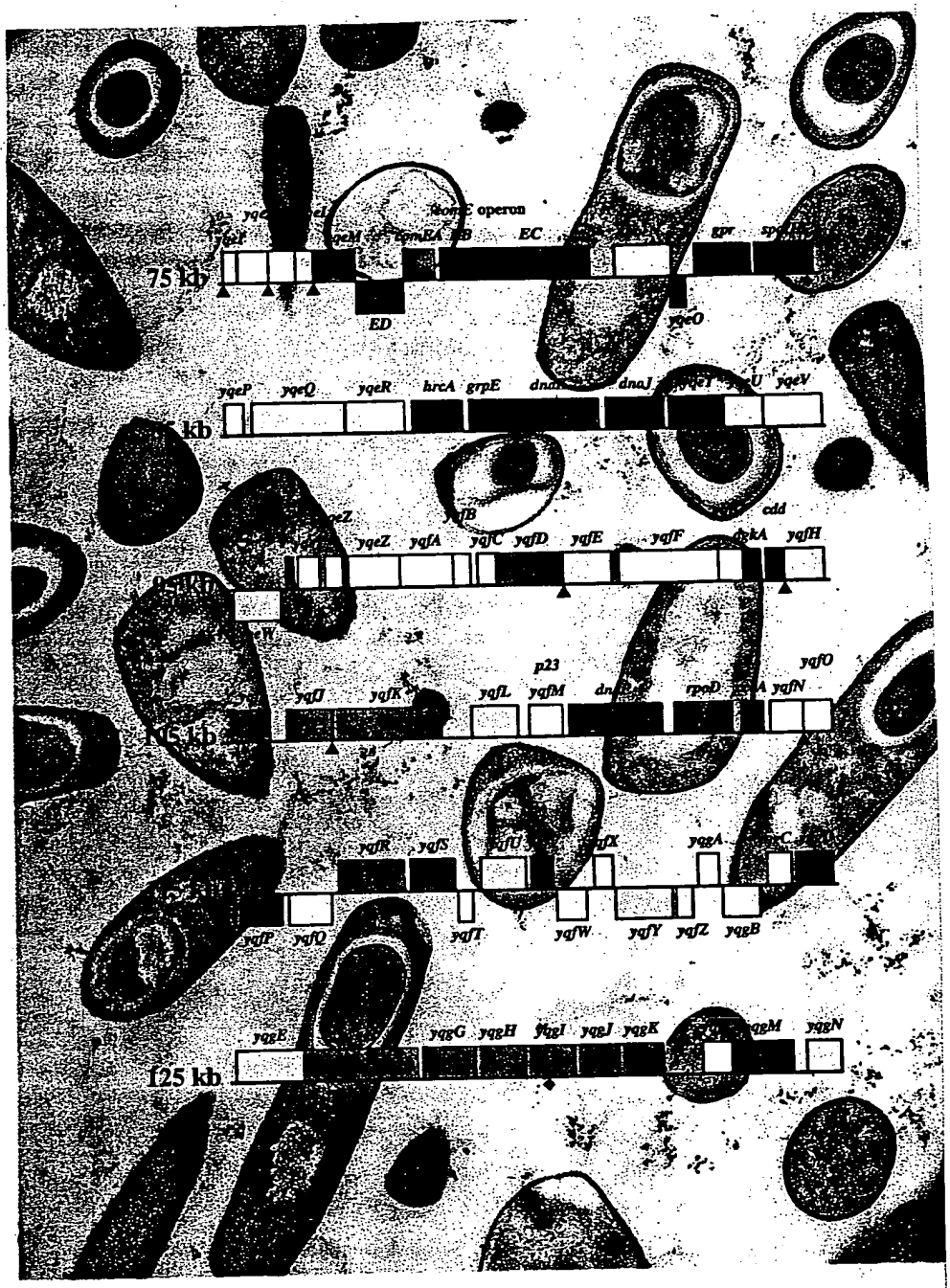

More from the *Bacillus subtilis* Genome Sequencing Project – about 80% is now complete

APPENDIX C

RELATED PROCEEDINGS APPENDIX TO APPEAL BRIEF UNDER RULE 41.37(C)(1)(X)

None